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# *Crop Biotechnology in the Service of Medical and Veterinary Science*

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A revolution in the basic science related to plants has occurred over the last fifteen years as techniques for genetic engineering have been developed. It is now possible to introduce foreign genes into almost all major crops, resulting in stable genetic transformants that pass the new trait on to their progeny following the principles of Mendelian genetics. Those protocols have led to detailed understanding of the mechanisms by which gene expression is regulated in the different tissues, and at different times, in the plant life cycle. From an applied standpoint, this understanding can be used to cause foreign genes to be expressed only in selected tissues — such as fruits or grains. Plant biotechnology has yielded commercial products in the field of agriculture as plants resistant to insects, viruses, fungi, and herbicides have been created. In addition, foods with modified ripening characteristics are now commercially available.

Beginning in the last few years, various research laboratories have experimented with the use of plants for "biomanufacturing" of specialty products. These approaches utilize transgenic plants created to accumulate high value proteins/enzymes. Some of those studies have explored the production of proteins of potential pharmaceutical value. It has been shown that plants will produce molecules as diverse as human serum albumin (which requires extensive conformation folding) and both serum and secretory antibodies (which require assembly of multiple peptide subunits to produce a functional antibody).

## Expression of Antigenic Proteins in Transgenic Plants

It has been well documented that molecular biology has had enormous impact on the fields of immunology and vaccine development. Tools to identify and clone the genes encoding antigenic determinants of infectious agents have been developed and are in wide use. The introduction of the gene encoding the surface antigen of the hepatitis B virus (HBV) in recombinant yeast was the first commercial example of a recombinant subunit vaccine. The HBV vaccine produced by recombinant DNA techniques is extremely safe and effective; however, its high cost has led the Institute of Medicine to list the development of a lower-cost recombinant DNA vaccine as a top priority. It can be readily anticipated that numerous other recombinant subunit vaccines will also become available in the next decade as our understanding of immune recognition of individual proteins grows. While these offer exciting opportunities for disease prevention, dependence upon fermentation technology and protein purification will influence both cost and availability of the new vaccines to the developing world. This led us to explore the use of transgenic plants as a subunit vaccine expression system.

## Hepatitis B Surface Antigen Expression in Plants

We began our studies of candidate vaccine expression in transgenic plants using the gene encoding hepatitis B surface antigen (HBsAg). This protein was chosen because the commercially available vaccine and the associated human immune response have been very well characterized, because the structure of the immunogenic form of that protein was known, and because the availability of a cost-effective recombinant HBV vaccine for the developing world is a high priority.

In our initial studies, the gene for HBsAg was introduced into cells of tobacco plants and individual transgenic plants were regenerated. We chose tobacco for the experiments because of its ease of genetic manipulation and abundant literature on controls for gene expression in this facile “model laboratory plant.” When transgenic leaf material was extracted, virus-like particles could be recovered. These were characterized and were found to be very similar in structural properties to the recombinant HBsAg, which is formulated in the commercial vaccine produced in yeast cells.

When plant-derived HBsAg was used for parenteral immunization of mice, anti-HBsAg antibodies were recovered that reacted with authentic HBsAg from human serum. This was our first indication that antigenic properties of the protein were maintained in recombinant plants. Subsequently, T-cells were isolated from mice immunized with tobacco-derived HBsAg. When grown in culture, these T-cells could be activated using the commercial vaccine, as well as a synthetic peptide that mimics the “a” epitope determinant of HBsAg. In total, the immunology studies conducted to date show that the recombinant HBsAg recovered from plant cells retains both B- and T-cell epitopes. These studies have demonstrated that plant cells have the capacity to not only synthesize this protein but to allow it to assemble in an immunologically active form.

## VACCINES AGAINST BACTERIAL DIARRHEAL DISEASE

Diarrheal disease is the major cause of infant mortality on a worldwide basis. Vaccines to prevent diarrheal disease caused by bacteria or viruses could have a significant impact on human health in the developing world. As my colleagues and I had an interest in testing the oral immunogenicity of recombinant antigens produced in plants, we made an early choice to focus on enteric diseases.

The binding subunit of the heat-labile enterotoxin of *E. coli* (LT-B) was an obvious candidate for evaluation in plant expression systems since it has been extensively characterized in structural and immunological studies. Because it is very similar in structure and immunological properties to the B-subunit of cholera toxin (CT-B), immunization with CT-B leads to cross protection against enterotoxigenic *E. coli* (ETEC). Early field studies of cholera vaccines in Bangladesh showed that CT-B immunization was useful in preventing cholera, although protection was relatively short lived and protective immunity would require frequent boosting.

Our initial studies utilized an expression vector that caused the production of recombinant LT-B in transgenic plants using the native bacterial gene. Characterization of this protein was hampered by the low amount of protein that accumulated in the plants. Subsequently, the bacterial gene was modified to encode a fusion protein, which has a six amino-acid microsomal retention signal at the C-terminus of LT-B. We reasoned that retention of the newly synthesized LT-B in microsomal vesicles of plant cells would allow an increase in the relative concentration of the protein, leading to assembly of the active pentameric form of LT-B. We found that higher amounts of LT-B accumulated, and it could be isolated for characterization. Based on its physical properties and its ability to bind $G_{M1}$ gangliosides, we determined that the LT-B fusion protein assembled into the active oligomeric structure within plant cells. When this protein was partially purified from transgenic plants and given by oral gavage to mice, both serum and secretory antibodies that were specific for LT-B could be recovered from the treated animals. These antibodies were effective in inactivating the *E. coli* toxin when tested using *in vitro* assays as an indicator of protective immunity.

My colleagues and I are continuing our efforts to create transgenic plants that accumulate abundant amounts of recombinant LT-B in edible tissues. We have been guided by the earlier CT-B vaccine field trials that used one milligram of protein per oral dose. By creating a synthetic gene that encodes an LT-B protein of the authentic amino acid sequence, but uses codons which are preferred by plants, we have recently been successful in creating plants that accumulate one milligram of LT-B in a potato that could be consumed raw by a human volunteer. We are cooperating with John Clements of the Tulane Medical School, as well as Michael Levine and Carol Tackett of the Center for Vaccine Development at the University of Maryland, to evaluate this material in human clinical trials for feasibility of use as an oral vaccine.

## VACCINES AGAINST VIRAL DIARRHEAL DISEASE

Multiple viruses cause diarrheal disease. Rotavirus may be the principal agent, especially in infants. As we began studies of the potential value of transgenic plants to produce recombinant subunit vaccines, I consulted with Dr. Mary Estes of the Baylor College of Medicine about the potential for a plant-based vaccine for rotavirus. From the data she provided, we concluded that coordinate expression of at least two (and possibly more) rotavirus coat proteins might be needed to cause the assembly of an immunogenic virus-like particle. Since we had not yet evaluated the capacity of transgenic plants to produce immunogenic proteins and because coordinate expression of multiple foreign proteins in plants had not been characterized, we concluded that a simpler system was needed that would provide "proof of principle." For this system we turned to the characterization of a candidate vaccine against Norwalk virus.

Norwalk virus (NV) is a member of the *Caliciviridae* family. It is a causal agent of severe epidemic outbreaks of viral diarrhea. When the gene encoding the single capsid protein of Norwalk virus was expressed in insect cells, virus-like particles (VLPs) could be isolated. These particles were also found to be effective in causing oral immunization of mice, resulting in production of both serum and antibody specific for the capsid protein. Unfortunately, there is no animal model to demonstrate protective immunity of vaccines against the Norwalk virus; those determinations will require human clinical trials.

Extracts of plants expressing the NV capsid protein were found to contain VLPs that mimicked the structural properties of VLPs recovered from insect cells. When these were partially purified and given to mice by gavage, both serum and secretory antibodies to the NV capsid protein were produced.

## ORAL IMMUNIZATION USING EDIBLE PLANT TISSUES

Although our initial subunit vaccine experiments used tobacco as a test system, our goal was to produce candidate vaccines in edible plant tissue to evaluate the potential for immunization simply by eating the tissue. Because the oral immunogenicity of plant-derived antigens had been demonstrated by the oral gavage studies, we developed protocols for genetic transformation of potato using genetic regulatory elements that cause the accumulation of the desired recombinant protein in the potato tuber tissues. We chose potatoes for two reasons. First, we could recover transgenic plants and grow them to maturity in greenhouses in a relatively short period (about three to four months). Secondly, mice will readily eat raw potatoes.

Transgenic plant material has been generated that contains both recombinant LT-B and the recombinant Norwalk virus capsid protein. The proteins assembled into the appropriate structures (LT-B pentamers that bound $G_{M1}$ gangliosides, or VLPs for the NV capsid protein). Potatoes were peeled, and five gram samples were fed to mice on a schedule that was analogous to the previous experiments using gavage for oral immunization. In both cases, the mice

produced serum and secretory antibodies against the recombinant protein in the potato eaten by the mice as food. These studies provide “proof of concept” for edible vaccines.

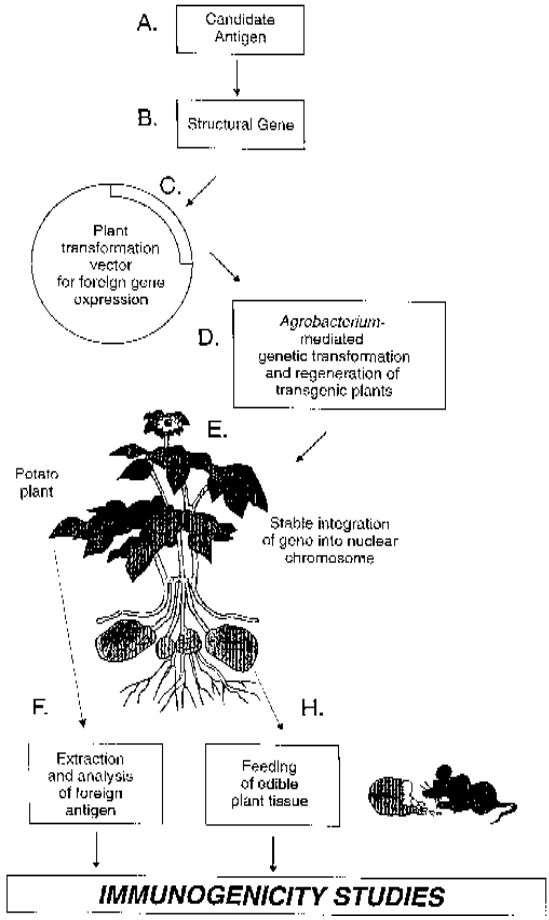


Figure 1. A schematic diagram showing the steps involved in creating transgenic plants expressing immunogenic proteins. A. Based upon earlier immunology studies, a candidate antigen with known or potential value as a subunit vaccine is identified. B. The structural gene encoding the antigen of interest is obtained or isolated. C. The gene is ligated into a plant transformation vector which contains plant-specific promoter and terminator sequences to regulate gene expression. D. The natural gene transfer system of *Agrobacterium tumefaciens* is usually utilized to mobilize the genetic construct into the chromosomes of plant cells. E. Plants are regenerated from transformed cells that contain a stably integrated gene encoding the desired antigen. F. and G. Immunogenicity of the plant-expressed antigen is determined by either extracting the foreign protein and delivering it by parenteral or oral routes, or simply by feeding a plant sample as food.

## Vaccines for Animal Diseases

Oral vaccines can provide efficient and humane strategies for disease prevention in production and companion animals, as well as feral populations. The above-mentioned "proof of concept" for edible vaccines suggests that the design of subunit vaccines in feed crops is a viable strategy. Seed-specific genetic regulatory elements are known for crops such as corn and soybeans, and stability of foreign proteins in dried seeds has been demonstrated.

Recently, transgenic plants have been generated that expressed the gene encoding the glycoprotein (G-protein), which coats the outer surface of the rabies virus. Although the immunogenicity of these materials has yet to be reported, it is encouraging to note that bait containing some G-protein produced in a more traditional in-vitro system was effective in orally immunizing raccoons, providing protection against "street virus" challenge.

## Remaining Questions/Opportunities for Edible Vaccines in Transgenic Plants

The research conducted to date has demonstrated that transgenic plants have the capacity to synthesize and accumulate subunit antigenic proteins that retain immunological properties of their native counterparts. In the case of the HBsAg and the NV-capsid protein, virus-like particles accumulate; this may be very significant because the particulate form of the proteins is likely to be important in determining immunogenic properties. It is likely that particulate structures will have greater oral immunogenicity than soluble proteins.

Studies not yet conducted will involve the evaluation of dosage requirements for plant-delivered vaccines. Successful experiments conducted thus far have used proteins from two enteric organisms. It will be necessary to determine if other proteins, which may not be normally transmitted via the oral route, will be as effective in inducing an oral immune response. We are optimistic, however, because plant cells represent a natural bioencapsulation system with surrounding layers of cell wall, cell membrane, and (in some cases) internal membrane compartments to encapsulate and thereby protect the desired subunit protein from digestive degradation. It remains to be determined if the release of the desired protein in the gut acts as a "slow release" as the plant cells are degraded in the normal digestive process. If so, dosage levels may have to be adjusted accordingly.

A potential major advantage of recombinant plants for vaccine production is the possibility that multi-subunit vaccines, including an oral adjuvant such as CT or LT (or derivatives thereof) could all be produced in a single plant. There is no theoretical limit to the number of different genes that could be introduced into a single plant species. Plant tissue could, therefore, contain multiple antigens in one delivery system. In addition, this could circumvent the need for a cold chain in vaccine delivery when produced and utilized in developing countries.

It is well recognized that most food proteins do not trigger an immune response; in general this is due to the induction of a state of immune tolerance. It will be necessary to determine if food-based vaccines will also induce oral tolerance to the desired antigen. If so, controlled use and dosage will be a requirement for "edible vaccines." This should not be an insurmountable obstacle, however, as only a small amount of plant material would need to be propagated for wide-scale vaccine delivery and its distribution could become a component of public health care systems.

The type of plant material that would best serve as an "edible vaccine" is yet to be determined. For human vaccines, our own research team has focused on the use of bananas. This crop has three major attributes: it is grown in almost all tropical or sub-tropical developing countries throughout the world; the food is eaten uncooked (which would avoid denaturation of subunit proteins); and bananas are a food that is widely consumed by infants and children. At the present time, we have developed a methodology for the genetic transformation of bananas and are cloning fruit-specific genetic regulatory elements, which we believe will cause the tissue-specific production of the desired candidate vaccine in the developing fruit. The primary disadvantage of bananas is one of technical limitations during feasibility stages of research. This is due to the fact that the time from genetic transformation until harvest and evaluation of the fruit is relatively long. We anticipate that the time period will be at least two years. Our first transgenic banana plants containing genes encoding candidate vaccines are still in the seedling stage.

For animal vaccines, a variety of grain crops (such as soybeans or corn) represent excellent possibilities for vaccine delivery. Transformation techniques for those crops are known, as are strategies for causing seed-specific gene expression.

In summary, the expression of vaccines in plants may be the first large-scale example of production of high-value pharmaceuticals in transgenic crops.

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